BCL-2 SPLICING VARIANTS

Field of the invention

This invention relates to transcripts of genes that encode regulators of mammalian cell viability and to the manipulation of cell viability through the targeting of variants of such transcripts.

Background to the invention

Mammalian cell viability is determined by a continual balance between pro- and anti-death signals. The best understood process is that of apoptopic cell death.

Bcl-2 is an inhibitor of apoptosis. The functions of the Bcl-2 protein include protection against mitochondrial changes associated with apoptosis. This is achieved by inhibiting pro-apoptotic proteins and by preventing mitochondrial permeability transition. Apoptosis can be triggered by release of cytochrome c and other pro-apoptotic components from the mitochondria: Bcl-2 is believed to inhibit such events. Consistent with these functions the Bcl-2 protein is predominantly localised to the mitochondria. Bcl-2 may also have additional anti-apoptotic functions yet to be described. It may also block mitochondrial-independent pathways involved in apoptosis.

The human Bcl-2 gene encodes mRNA transcripts of (i) 720 nucleotides in length for Bcl-2α and (ii) of 618 nucleotides in length for Bcl-2β (see Figure 1). Bcl-2α and Bcl-2β represent normal, alternatively spliced variants of the same Bcl-2 gene. Abnormal and/or constitutive expression of functional Bcl-2 can protect mammalian

cells from undergoing apoptosis. Such an effect favours continued cell survival and proliferation, and can initiate and/or maintain abnormal and/or cancerous growth.

In colorectal cancer cells evidence for a novel Bcl-2 – p53 axis has been reported for a number of established human colorectal carcinoma cells lines, including the LoVo and SW48 cell lines. Co-pending patent application GB0306148.8 relates to the silencing of Bcl-2 by RNA interference. p53-dependent apoptosis is induced indicating that Bcl-2 constitutively suppresses a pro-apoptotic function of p53 in colorectal cancer cells. Importantly, this pro-apoptotic function of p53 does not require activation of the p53 protein by genotoxic stress or by other means. Constitutive Bcl-2 suppression of p53-dependent apoptosis is likely to contribute to the survival of human colorectal cancer cells.

There is a need to identify cell growth control targets for treating malignancies in humans and other mammalian species.

Statements of the invention

According to the present invention there is provided a method of regulating apoptosis in a cell, said method comprising targeting an abnormally or alternatively spliced mRNA, an abnormally or alternatively structured mRNA, or a product of either.

The term 'regulate' is used to refer to the situation where the threshold of apoptosis in a cell is controlled or adjusted to a particular specification or requirement and may refer to either 'up regulation', wherein the threshold of apoptosis is increased as

compared to that which is observed in said cell, in absence of performance of the method, or down regulation, where the threshold of apoptosis is decreased as compared to that which is observed in said cell, in absence of performance of the method.

Within genes, DNA serves as a template for the production of messenger RNA, which in turn is a template for the production of proteins. Messenger RNA molecules typically contain protein-coding regions called "exons" as well as non protein-coding regions called 'introns'.

It is known that mammalian RNA transcripts are modified in the nucleus by additions to the 5' and 3' ends of the molecule and by internal splicing to remove the introns. The splicing event requires breakage of the exon-intron junctions and joining of the ends of the exons. By comparing the nucleotide sequence of mRNA with that of the structural gene, the junctions between exons and introns can be assigned. The junctions have well conserved, though rather short consensus sequences. The really high conservation is found only immediately within the intron at the presumed junctions. An intron starts with the dinucleotide GT and ends with the dinucleotide AG. Accordingly, the junctions are often described as conforming to the GT-AG rule. The GT-AG rule describes the splicing sites of nuclear genes of many (perhaps all) eukaryotes.

However, the above is a very simplistic view of gene splicing. With the advent of information generated by various genome sequencing programmes it is evident that alternative pre-mRNA splicing is frequently used to expand the coding capacity of genomes. Splicing motifs are being continually discovered and tissue specific splicing patterns are emerging. Exonic splicing silencers (ESS) are exonic cis-regulatory elements that inhibit splicing, often leading to exon skipping. Thus the permutations of genetic information expressed via a single gene can be amplified and regulated.

The Bc1-2 gene encodes 3 exons. The interspersed introns must be precisely snipped away from Bcl-2 messenger RNA and the remaining exons must be accurately spliced together, with no 'exon skipping', if a normal Bcl-2 protein is to be produced. The splicing machinery in the cell nucleus cuts and pastes (due to a "lariat" intermediate) to generate a single, properly spliced Bcl-2 messenger RNA molecule. Normally two alternative splice variants are detected, Bcl-2α and Bcl-2β, to give protein products of 239 and 205 amino acids respectively.

Exon skipping due to mutations in Bcl-2 and many other genes is frequently, if not always, caused by the disruption of "exonic splicing enhancers," or ESEs. ESEs are sequences within exons that stimulate messenger RNA splicing. Diverse mutations in genes lead to RNA splicing defects and in turn, to various diseases.

The term 'abnormally or alternatively spliced' is used interchangeably to refer to the situation where mRNA is spliced using a splice sequence not normally used in processing the normal transcript(s) and the resulting mRNA sequence is different to that of the full-length normal sequence transcripts. Internal structures within the mRNA transcript, for example stem loops and pseudo knots, can also affect the information flow from transcript to translated protein product.

Preferably the method involves targeting the junctions of mRNA molecules that are abnormally or alternatively spliced or abnormally or alternatively structured.

The term 'junction' is used to refer to the particular nucleotide sequence that is created by the attachment or joining together of the alternatively spliced mRNA. The term 'junction' also encompasses the apparent junction created by the presence of secondary RNA structures within the mRNA: such structures can cause looping out from a lateral stem on the mRNA such that the apparent linear sequence resembles that of a spliced junction upon amplification by RT-PCR. For Bcl-2 mRNA from colorectal carcinoma cells evidence for such structures has been discovered using Abgene reverse blender RT-PCR amplification at 47°C; and the Two-step Sigma RT-PCR kit at 47°C. Both these procedures give shorter spliced Bcl-2 cDNAs and, importantly, the apparent splicing conserves the triplet reading frame for Bcl-2 mRNA (Figure 1). However, when RT-PCR is carried out using Quiagen sense-script reverse transcriptase at 50°C, the cDNA product is for full length Bcl-2 mRNA. These results indicate that Bcl-2 mRNA from colorectal cancer cells contains highly ordered loop structures.

Alternatively the method involves targeting a protein product following translation of an abnormally/alternatively spliced mRNA or abnormally/alternatively structured mRNA.

Preferably the method comprises selective silencing of abnormal splice variants of the Bcl-2 gene.

The term 'selectively silencing' is used to indicate that the silencing is specific for the target gene and that there is no interference with normal, endogenous gene expression which might be detrimental to normal non-cancerous cells.

Preferably the method involves the targeting of any of the abnormal splice variants Bcl-2α-591, Bcl-2α-588, Bcl-2α-480, Bcl-2α-633, Bcl-2β-489, Bcl-2β-474, Bcl-2β-420 and/or Bcl-2β-315. More preferably the method involves targeting the mRNA sequence flanking the splice junction between nucleotides 111 and 241 of Bcl-2α-591.

In one embodiment the method of the invention involves targeting an abnormally/alternatively spliced or abnormally/alternatively structured mRNA or a product of either, by introducing into a cell containing a gene which is abnormally spliced and which is to be targeted, an RNA construct having a nucleotide sequence which is homologous to mRNA within said cell wherein said mRNA includes genetic information of the gene element that is abnormally spliced.

Is is known that the introduction of dsRNA into cells initiates RNA interference (RNAi). RNAi induces sequence-specific degradation of homologous mRNA. In mammalian cells RNAi can be achieved using small interfering dsRNAs (siRNAs), preferably up to 28 nucleotides long and more preferably 21-22 nucleotides long.

The term 'homologous' is used to indicate at least 50%, preferably 85%, more preferably 90%, more preferably 95% and most preferably 100% homology to the reference nucleic acid sequence.

The present invention relates to the discovery of abnormal splice variants of Bcl-2 mRNA in human colorectal carcinoma cells. Sequence alignments are given in Figure 1. The novel splice junctions conserve the normal triplet framing of the spliced mRNA products and the functional BH1, BH2, BH3 and BH4 domains of the Bcl-2 protein are also conserved (where BH stands for 'Bcl-2 homology domain').

Abnormal alternatively spliced variants of Bcl-2 may function constitutively to suppress apoptosis in human and other mammalian cells, enabling abnormal cell survival and abnormal cell proliferation. The expression of abnormally spliced variants of Bcl-2 may thus represent a key oncogenic event in the development of cancer. The abnormal splice junctions of the Bcl-2 mRNA molecules represent selective targets for intervention via RNA interference or other means. The mRNA sequence at these abnormal splice junctions is not present in the normally spliced full length Bcl-2 mRNAs.

These abnormal Bcl-2 mRNA transcripts are shorter than the full length 'wild type' Bcl-2 mRNA. In contrast analysis of the genomic Bcl-2 by PCR amplification gives the predicted length for wild type Bcl-2 DNA (Figure 2). This indicates that the shorter abnormal Bcl-2 mRNA transcripts are indeed generated by alternative splicing of RNA, rather than genomic events with loss of DNA coding sequence from the human Bcl-2 gene.

The abnormal alternative spliced variants of Bcl-2 mRNA expressed in human colorectal cancer cells remain in-frame for the triplet genetic code and retain all known functional domains of the Bcl-2α and Bcl-2β proteins (see Figure 1) and are functional in the suppression of apoptosis. Functionality is also evident in colorectal carcinoma cell lines in which Bcl-2 expression may comprise solely of the abnormal alternative mRNA form(s). In such cells the selective silencing of Bcl-2 expression by RNA interference induces apoptosis (Jiang and Milner, 2003).

In one embodiment of the invention, selective silencing of alternatively spliced Bcl-2 expression is achieved by RNA interference. Alternatively silencing may be achieved by any other 'silencing means' such as small molecules, peptides and/or related molecules that inhibit Bcl-2 either directly or indirectly, and also Bcl-2 derived products including abnormal Bcl-2 splice variants. Anti-sense RNA, shRNA, miRNA and any other RNA and/or DNA based strategies may also be used. Tumour cells other than colorectal cancer cells may similarly be treated, such as ovarian cancer cells.

In one embodiment the present invention provides a nucleotide construct with a nucleotide sequence that is homologous to mRNA transcribed from an abnormally or alternatively spliced gene.

Preferably the nucleotide construct comprises dsRNA. Preferably the construct is 30 or less nucleotides long. More preferably the RNA construct is 20 to 30 nucleotides long. Most preferably the RNA construct is 21 to 22 nucleotides long.

In one embodiment the invention provides a nucleotide construct such as anti-sense RNA, shRNA or miRNA as means for silencing the expression of an abnormally or alternatively spliced gene for use as a medicament.

In an alternate embodiment the invention provides an agent selected from the group consisting of: small molecule or protein, polypeptide; peptide; aptamer; chemical; antibody; nucleic acid; polypeptide or nucleotide probe which agent interacts with or binds with a protein expressed by an abnormally spliced mRNA for use as a medicament. Preferably the agent comprises a sequence or molecular structure that is complimentary to or of sufficient homology to give specific binding to the target.

In an alternative embodiment the invention provides a nucleotide construct such as anti-sense RNA, shRNA or miRNA for the manufacture of a medicament for the treatment of cancerous cell growth.

In an alternate embodiment the invention provides an agent selected from the group consisting of: small molecule or protein, polypeptide; peptide; aptamer; chemical; antibody; nucleic acid; polypeptide or nucleotide probe which agent interacts with or binds with a protein expressed by an abnormally spliced mRNA for the manufacture of a medicament for the treatment of cancerous cell growth.

The invention also provides a pharmaceutical composition comprising a nucleotide construct such as anti-sense RNA, shRNA or miRNA and a pharmaceutically acceptable diluent or carrier.

In an alternate embodiment the invention provides an agent selected from the group consisting of: small molecule or protein, polypeptide; peptide; aptamer; chemical; antibody; nucleic acid; polypeptide or nucleotide probe which agent interacts with or binds with a protein expressed by an abnormally spliced mRNA and a pharmaceutically acceptable diluent or carrier.

According to a further aspect of the invention there is provided a DNA or RNA expression vector as a delivery means for, for example, an antisense or an RNAi molecule that is used in the targeting of an abnormally spliced mRNA or a product thereof.

In one embodiment of the invention a viral vector is used as delivery means.

Preferably the vector includes an expression cassette comprising the nucleotide sequence selected from the group consisting of;

a) the nucleic acid sequence of the abnormally spliced gene element as shown in Fig 1;

b) a nucleic acid molecule which hybridizes to the nucleic acid sequence of (a) ;

c) a nucleic acid molecule which has a nucleic acid sequence which is degenerate because of the genetic code to the sequences in a) and b) and any sequence which is complimentary to any of the above sequences;

wherein the expression cassette is transcriptionally linked to a promoter sequence.

Preferably the vector including the expression cassette is adapted for eukaryotic gene expression. Typically said adaptation includes, by example and not by way of limitation, the provision of transcription control sequences (promoter sequences) which mediate cell/tissue specific expression. These promoter sequences may be cell/tissue specific, inducible or constitutive.

Promoter elements typically also include so called TATA box and RNA polymerase initiation selection sequences which function to select a site of transcription initiation. These sequences also bind polypeptides that function, *inter alia*, to facilitate transcription initiation selection by RNA polymerase.

Adaptations also include the provision of selectable markers and autonomous replication sequences which both facilitate the maintenance of said vector in either the eukaryotic cell or prokaryotic host. Vectors that are maintained autonomously are referred to as episomal vectors. Further adaptations which facilitate the expression of vector encoded genes include the provision of transcription termination sequences.

These adaptations are well known in the art. There is a significant amount of published literature with respect to expression vector construction and recombinant DNA techniques in general. Please see, Sambrook et al (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbour Laboratory, Cold Spring Harbour, NY and references therein; Marston, F (1987) DNA Cloning Techniques: A Practical Approach Vol III IRL Press, Oxford UK; DNA Cloning: F M Ausubel et al, Current Protocols in Molecular Biology, John Wiley & Sons, Inc. (1994).

Detailed Description of the Invention

The present invention will now be described by way of example only and with reference to the following diagrams;

Figure 1

Sequence alignments of human Bcl-2 splice variants in colorectal cell lines (including LoVo; SW48 and HCT116). Boxed areas indicate functional domains of Bcl-2. Note that Bcl-2α-591; -α588; -α480; -α633 and Bcl-2β-489; -β474; -β420 and –β315 retain all functional domain sequences. Dashes indicate missing sequences from abnormally spliced Bcl-2 variants.

Figure 2

Sizing of Bcl-2 genomic DNA following PCR amplification from individual human colorectal cell lines as indicated, using primers designed to span all abnormal splice sites identified to date. The predicted size for the intact genomic Bcl-2 DNA PCR-generated sequence, using the chosen primers, is 570 base pairs. This is the size observed in all colorectal cell lines tested to date, as indicated on the gels. [Note that genomic Bcl-2 is normally only spliced to generate the Bcl-2α and Bcl-2β variants].

Figure 3

Expression of abnormal alternatively spliced variants of human Bcl-2 in vitro and immunoprecipitation with anti-Bcl-2 antibodies. Bcl-2 mRNA from human colorectal cancer cells was reverse transcribed to produce a cDNA template from which cRNA was transcribed and translated. Translation was performed in the presence of 35S-methionine and radiolabelled protein was visualised by autoradiography following immunoprecipitation and resolution by SDS-PAGE. Three abnormal splice variants are shown (Bcl-2α-591; Bcl-2β-489; and Bcl-2β-420 as indicated).

Figure 4

Table summarising the truncated RNA products derived from the Bcl-2 gene and detected by PCR in samples of different colorectal cell lines. HCT = HCT116 with six individual clones which fall into three isogenic pairs with knock-out for p53; p21 and Bax genes, as indicated.

Figure 5

Radiolabelled Bcl-2 proteins following in vitro transcription and translation using rabbit reticulocyte lysate. Proteins were resolved by SDS-PAGE and visualised by autoradiography. Upper band = full length Bcl-2α; lower bands = protein products generated via alternatively spliced/alternatively structured Bcl-2 mRNA.

Materials and Methods

Bcl2 detection and cloning by RT-PCR

The primers used for Bcl2 amplification in colon cancer cell lines were as follows:

	5'→ 3'
Bcl-2up	ccatcgatggcgcacgctgggagaac
Bcl-2dn(α)	ccggaattcacttgtggcccagatagg
Bcl-2dn(β)	ccggaattcagcccagactcacatcacca
Bcl-2up2	ccgggagatagtgatgaagtaca
Bcl-2dn2	cctggatccaggtgtgcaggt
Bcl-2dn3	tgccggttcaggtactcagtc

The RT-PCR is performed using 100ng total RNA, bcl2up and bcl2dn with one-step RT-PCR kit from ABgene (cat. AB-0844 or AB-0844/b) in a thermal cycle as follows: 47°C 30min, 94°C 2min, then 35 cycles of 94°C, 45sec, 58°C, 45sec, 72°C, 1min, followed by 72°C for 5min. There are only shortened Bcl-2 products amplified using above method. The full-length Bcl-2 product can only be amplified using Qiagen one-step RT-PCR kit in a thermal cycle as below: 50°C 30min, 94°C 15min,

then 35 cycles of 94°C, 45sec, 58°C, 45sec, 72°C, 1min, followed by 72°C for 10min.

The PCR products were purified and digested with *EcoR*I and *Cla*I, and cloned into pBSK$^+$, then transcribed using T7 polymerase and translated using Promega RRL in vitro. The translated protein were immuno-precipitated with various Bcl-2 antibodies: BD (BD biosciences), C-2, N-19 (Satan Cruz), Ab-1, Ab-2, and Ab-4 (Oncogene).

Bcl-2 antibodies employed in this study – positions of their epitopes:

Bcl-2 (BD) Against 49-179aa. From current study, it can be refined to 81-88aa.

Bcl-2 (C-2) Against a recombinant protein corresponding to amino acids 1-205. From the current study, it can be refined to 81-88aa.

Bcl-2 (N-19) Against a peptide mapping at the amino terminus of Bcl-2. From the current study, it can map at 1-23aa.

Bcl-2 (Ab-1) 41-54aa.

Bcl-2 (Ab-2) 20-34aa.

Bcl-2 (Ab-4) 61-76aa.

Cloning and expression of abnormal alternative splice variants of Bcl-2 in vitro.

Abnormal alternative splice/structural variants of Bcl-2 mRNAs have been cloned from colorectal cancer cells and expressed in vitro. The results demonstrate that the abnormal alternative splice/structural variants of Bcl-2 are expressed as protein (Figure 3).

Lack of specific Bcl-2 epitopes was observed for the protein products encoded by the abnormal alternatively spliced Bcl-2 variants. Abnormal splicing in some way interferes with epitope availability for antibody recognition. It is proposed that epitope loss may prove to be a useful indicator of alternatively spliced Bcl-2 expression. For example, the variant Bcl-2α-591 appears to contain a novel splice junction between nucleotides 111 and 241 (Figure 1): the protein expressed endogenously from this splice variant in human cells reacts poorly with the N19 anti-Bcl-2 antibody in immunoblots (Jiang and Milner, 2003), and in immunoprecipitation reactions following its expression in vitro (Figure 3). Loss of antibody reactivity may also be evident in tissue sections stained by immunocytochemistry. Epitope loss or modification may prove to be of clinical and diagnostic importance for identifying the expression of abnormal alternative spliced variants of Bcl-2 in human tissues. The same principles apply to tissues of other mammalian species.

Alternative abnormal spliced variants of Bcl-2 may represent a tumour-related abnormality. This abnormality may not be restricted to cancers arising from

colorectal epithelial cells. Other tumour types may also be affected, including other epithelial tumours and/or tumours/malignancies arising from other cell types. Any tumour-related abnormality represents a promising target for selective therapy designed to selectively target malignancies in humans and in other mammalian species. Such therapies may, in principle, be designed to suppress gene expression using, for example, RNA interference. An alternative approach would be to target abnormal mRNA structures using selective binding molecules. An alternative approach would be to target functional protein-protein interactions by, for example, small molecules designed to disrupt essential molecular interfaces between the Bcl-2 protein and its functional protein partners. Any differences in protein structure created as a result of abnormal alternative splicing of Bcl-2 mRNA represent potential tumour-specific targets for novel anti-cancer molecules and/or other reagents.

References:

1. **Jiang M & Milner J.** Bcl-2 constitutively suppresses p53-dependent apoptosis in colorectal cancer cells. **Genes & Development,** 17; 832-837 (2003).